iTRAQ-Based Quantitative Proteomics Analysis of Sprague-Dawley Rats Liver Reveals Perfluorooctanoic Acid-Induced Urea Metabolism Dysfunction

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Abstract

Perfluorooctanoic acid (PFOA) is a typical C8 representative compound of perfluoroalkyl and polyfluoroalkyl substances (PFAS) widely used in industrial and domestic products. It is a persistent organic pollutant found in the environment as well as in the tissues of humans and wildlife. Despite emerging scientific and public interest, the precise mechanisms of PFOA toxicity remain unclear. In this study, male rats were exposed to 1.25, 5, and 20 mg PFOA/kg body weight/day for 14 days. Urine samples were also collected and monitored by raising rats in metabolic cages. *In vivo* results demonstrate that PFOA exposure induces significant hepatocellular hypertrophy and reduced urea metabolism. iTRAQ-based quantitative proteomics analysis of Sprague-Dawley (SD) rats livers identified 3,327 non-redundant proteins of which 112 proteins were significantly upregulated and 80 proteins were downregulated. Gene ontology analysis revealed proteins are primarily involved in cellular, metabolic and single − organism processes. Among them, eight proteins (ACOX1, ACOX2, ACOX3, ACSL1, EHHADH, GOT2, MTOR and ACAA1) were related to oxidation of fatty acids and two proteins (ASS1 and CPS1) were found to be associated with urea cycle disorder. The downregulation of urea synthesis proteins ASS1 and CPS1 after exposure to PFOA was then confirmed through qPCR and western blot analysis. Together, these data demonstrate that PFOA exposure directly influences urea metabolism and identify CPS1 as a potential regulatory target.

Introduction

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) are a class of synthetic chemicals that are increasingly recognized as a new type of persistent organic pollutants (POPs). POPs contain high-energy C-F covalent bonds in which all hydrogen atoms may be replaced by fluorine atoms. The archetypal PFAS, perfluorooctanoic acid (PFOA), is a perfluorocarboxylic acid with 8 C atoms. The fluorine-containing special structure of PFOA is responsible for its hydrophobicity, oleophobicity and extremely low surface tension. As such, PFOA is widely used in various commercial and industrial settings including manufacture of textiles, packaging materials, surfactants, pharmaceuticals and fire-extinguishing foam (Convertino et al. 2018; Preston et al. 2018). The widespread use of PFOA has led to the mass production and emission of such compounds. For example, from 1951 to 2015 the global emissions from the production and use of perfluorocarboxylic acid reached 2,610 − 24,100 tons and it is estimated that from 2016 to 2030, the world will continue emission of 20 − 6,420 tons of C4-C14 perfluorocarboxylic acid (D'Agostino and Mabury 2017; Gingerich and Mauter 2017; Meng et al. 2017). In addition, the C-F high-energy covalent bond contained in PFOA is an extremely stable chemical bond that allows PFOA to persist in the environment (Cui et al. 2018; Hu et al. 2018). At present, PFOA has been detected in almost all environmental media (Wei et al. 2018; Yao et al. 2018), even in polar regions (Yeung and Dassuncao 2017). Due to its limited biodegradability, accumulation of PFOA has also been detected in birds and mammals (Rigét et al. 2013; Rotander et al. 2012) as well as in multiple reports of accumulation in human serum samples (Gao et al. 2018; Gebbink et al. 2015; Wang et al. 2018).

In recent years, an increasing number of studies have revealed the toxic effects of PFOA accumulation in organisms (Andersen et al. 2008; Kennedy et al. 2004; Lau et al. 2007; Lau et al. 2004). In general, PFOA accumulation interferes with cellular lipid metabolism, leading to carcinogenicity, liver toxicity, developmental toxicity, immunotoxicity, endocrine interference and neurotoxicity. Previous reports from our lab have demonstrated that PFDoA exposure can restrict amino acid metabolism in rats and thereby influence the synthesis of urea (Liu et al. 2016). In the following study, iTRAQ-based quantitative proteomics was utilized to screen for global proteomic profile alterations in rat livers after exposure to PFOA. We hereby demonstrate the differential expression of *ASL1, ASS1* and *CPS1* and identify their role as PFOA sensitive genes related to the urea cycle. These findings clarify the potential mechanisms responsible for PFOA toxicity *in vivo* and provide reference targets for future intervention and treatment of PFOA accumulation in humans.

Materials And Methods

Chemicals

PFOA (CAS No. 335-67-1, 95% purity) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Stock solutions of PFOA (0.01 M) were prepared in distilled Milli-Q water. Working solutions were prepared by serial dilution with distilled Milli-Q water. iTRAQ reagents are purchased from ABI (iTRAQ Reagent-8 Plex Multiplex Kit, AB Sciex, USA). Na_2CO_3, sodium orthovanadate, sodium pentobarbital, PBS and paraformaldehyde are purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), Other reagents are AR grade.

Animal treatments

40 male SPF grade, Sprague-Dawley rats (200 ± 10 g) were obtained from Qinglongshan animal breeding company (Nanjing, China) at 6–8 weeks of age. The rats were maintained in a SPF grade facility on a 12-h light/12-h dark cycle and were allowed *ad libitum* access to a standard diet and pure water. The ambient temperature in the animal room was 23 ± 1℃ and the relative humidity was 60 ± 5%. After one week of adaptation, the rats were randomly separated into four groups of 10. The treatment rats were given doses of 1.25, 5, and 20 mg PFOA/kg body weight/day by oral gavage for 14 consecutive days. The control animals were also treated with Milli-Q water, accordingly. At the end of the experiment, 7 rats from each group were weighed and anesthetized with sodium pentobarbital (45 mg/kg). Afterwards, blood was drawn from the inferior caval vein, and liver tissues were rapidly collected, weighed, rinsed with PBS, divided into small aliquots, flash frozen in liquid nitrogen before being stored at -80℃ until further analysis. The remaining three rats from each group were used for Clinicopathologic analysis. All procedures were performed in accordance with the Ethics Committee of Bengbu Medical College, Anhui Province.

Urine collection and analyses

10 rats were randomly divided into 2 groups: high dosage group and normal control. Rats were raised in metabolic cages to collect urine samples of 24 h volume for 14 consecutive days. Urea concentration

was determined by using commercially available kits (Nanjing Jianchen Bioengineering Institue, China).

Histopathological Examination

Three livers from each group were fixed in freshly prepared paraformaldehyde (3.7% in DPBS) and processed sequentially in ethanol, xylene and paraffin. Tissues were then embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin (HE stains).

Serum Biochemistry Analysis

Serum levels of 5'-nucleotidase (5-NT), adenosine deaminase (ADA), albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin direct (BILD), total bilirubin (BILT), total cholesterol (TCHO), creatinine (CREP), gamma glutamyl transferase (GGT), high density lipoprotein cholesterol (HDL_C), lactic dehydrogenase (LDH), low density lipoprotein cholesterol (LDL_C), total bile acid (TBA), total protein (TP), triglyceride (TG), Urea (UREA), Uric acid (UA) and creatinine (CR) were measured using cobas® 8000 modular analyser series (F. Hoffmann-La Roche Ltd).

Protein Preparation, iTRAQ Labeling

3 individual liquid nitrogen frozen livers from normal control rats and three PFOA treated livers from 20 mg PFOA/kg/d group were randomly selected for iTRAQ based mass spectrometry analysis. Proteins were extracted by dissolving each liver sample in 300 μL of ice-cold 0.1 M Na_2CO_3 and 10 mM sodium orthovanadate (pH 11) supplemented with protease inhibitor (Roche Complete EDTA Free) and phosphatase inhibitor (Roche), sonicated for 3 × 10 seconds and stored on ice. The bicinchoninic acid assay (BCA assay) was used to measure 200 μg proteins and mixed with urea/thiourea denaturation buffer to a final concentration at 6 M urea, 2 M thiourea. All protein samples were trypsinized (mass spec grade, Promega). The tryptic peptides in the three biological samples from the control and PFOA-treated groups were labeled with iTRAQ reagents (isobaric tags 115, 116, and 117 for the control; 118, 119 and 121 for the treated group) (iTRAQ Reagent-8 Plex Multiplex Kit, AB Sciex). The iTRAQ labeling was performed according to the manufacturer's protocol.

LC -MS/MS analysis

Mass spectroscopic (MS) analysis was performed using an Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer (Thermo Scientific, USA) and coupled with an EASY-nLC HPLC system (Thermo Scientific, USA). The iTRAQ labeled peptieds were loaded onto a C18-reversed phase column (3 μm-C18 resin, 75 μm ×15 cm) and separated on an analytical column (5 μm-C18 resin, 150 μm × 2 cm; GmbH, Ammerbuch, Germany) using mobile phase Buffer A: 0.5% formic acid / H_2O and Buffer B: 0.5% FA/ACN at a flow rate of 300 nL / min, using a 150 min gradient. Spectra were acquired in Data Dependent Acquisition (DDA) mode.

Database search for peptide and protein identification

The raw mass data were analyzed using Thermo Proteome Discoverer version 1.4 (ver. 1.4.0.288; Thermo Fisher Scientific) and with a false discovery rate (FDR) < 1% and expected cutoff or ion score < 5% (with

95% confidence) for searching the Uniprot Rat Complete Proteome database. The following options were used to identify the proteins: Peptide mass tolerance = ± 10 ppm, MS/MS tolerance = 0.6 Da, enzyme = trypsin, missed cleavage = 2, fixed modification: iTRAQ 8plex (K) and iTRAQ 8plex (N-term), variable modification: oxidation (M), database pattern = decoy.

GO annotation and KEGG pathway analysis

To analyze the differentially expressed proteins in PFOA treated group compared with normal control group, Gene Ontology (GO) annotation of the identified proteins was performed by searching the GO Web site (http://www.geneontology.org) to catalog the molecular functions, cellular components, and biological processes. Protein interactions and biological pathways were determined using the ResNet database (version 6.5, Ingenuity Systems, Inc.) (KEGG) to better understand these differentially expressed proteins in relation to the published literature.

RNA Isolation and Quantitative real-time PCR

Rat livers were used for RNA extraction and subsequent qPCR assays. Total RNA of the liver samples was isolated using a Trizol reagent (Ambion, Thermo Fisher Scientific, USA) and the isolation process was performed according to the manufacturer's instructions. Quantitative real-time PCRs (qPCR) were performed on a QuantStudio 3 Real-Time PCR System (Thermo Scientific, USA) using a SYBR Green Real Master Mix Rox (Tiangen, China). The housekeeping gene *GAPDH* was used as an internal control. The information of the primer pairs are listed in the supplementary table S1. The relative quantification of target genes was calculated based on the $2^{-\Delta\Delta CT}$ method.

Western blotting authentication

Protein extracts from the control and PFOA exposure group liver tissues were used for western blot analysis. The western blot is briefly as follows: Total proteins from liver of each rats were extracted with RIPA (Thermo Scientific, USA) containing 1 mM PMSF (Sigma-Aldrich, USA) and 1% phosphatase inhibitor (Sigma-Aldrich, USA). The protein concentration was determined by using a BCA kit (cwbiotech, China). Approximately 40 μg of total protein was loaded on 10% sodium dodecyl sulfate (SDS)-polyacrylamide-gels and then transferred to polyvinylidene fluoride membrane (PVDF) tansblot membranes (Amersham Biosciences, Piscataway, NJ, USA). The blotted membranes were blocked in blocking buffer (TBST) for 1 h, and then incubated with primary antibodies dissolved by blocking buffer on a shaker overnight at 4°C. (The information of the primary antibodies is listed in supplementary table S2.) After washing with TBST for 3 times, the membranes were then incubated with fluorescent-conjugated anti-rabbit IgG as the secondary antibody for 1 h at room temperature, respectively. The immunoreactive bands were photoed and analyzed by Gel Doc XR + Gel Documentation System (BIO-RAD, USA).

Statistical analyses

Data were analyzed using SPSS for Windows 17.0 Software (SPSS, Inc., Chicago, IL) and presented as means with standard errors (mean ± SE). Differences between the control and treatment groups were

determined using one-way analysis of variance (ANOVA). A P value of < 0.05 was considered statistically significant. OriginPro 2018 software was used to develop graphs (Origin Lab Corporation, USA).

Results

PFOA cause liver damage and influence urea synthesis

After 14 days, we find that PFOA exposure may cause body weight loss and significant liver swelling (Fig. 1A-B). Both absolute and relative liver weight were significantly increased by PFOA exposure. HE-stained liver slices from PFOA exposed mice also show significant liver swelling (Fig. 1C). In order to quantify the cell size, we counted the number of cells per unit area. The results show that the number of nuclei per area is significantly reduced following PFOA exposure (Fig. 1D); furthermore, this phenomenon has a clear dose-effect relationship with PFOA concentration.

We also monitored the effects of PFOA on metabolism of rats. 14 days of PFOA exposure had no significant influence on daily food intake (Fig. 2A). However, PFOA exposure had significant effect on urea metabolism. Rats exposed with PFOA had significantly lower urea concentration in urine compared with normal control rats (Fig. 2B).

PFOA has effects on sera biochemical parameters

To investigate the effect of PFOA on urea metabolism, we assayed 19 biochemical indexes in rat serum using a cobas® 8000 modular analyser series automatic biochemical analyzer. The results show that 8 indexes have significant changes compared with normal control group (Table 1). These indexes are ALT, ALT/AST, ALP, Urea, TG, TC, HDL-C, LDL-C. In clinical biochemistry, ALT, ALT/AST, ALP are closely related to liver function, serum urea is one of the main indicators of renal function, and TG, TC, HDL-C, LDL-C are closely related to liver lipid metabolism. The levels of ALT, ALT/AST, which are important biochemical indicators of liver damage, were significantly increased in the 20 mg/kg/d group. The other indexes which showed no significant changes are listed in supplementary table S3.

While the urea content of urine was significantly decreased in PFOA treated rats compared to normal rats, the level of urea in the serum of the 20 mg/kg/d treatment group was significantly increased.

Differentially Expressed Protein Identification and Relative Quantification by iTRAQ Analysis

Three individual samples were included in the iTRAQ experiment from the control and 20 mg PFOA/kg/d group. The MS/MS analysis identified a total of 25, 506 unique spectra matched to special peptides. Proteome Discover version 2.1 identified a total of 8,369 unique peptides from 2,868 proteins.

Bioinformatics Analysis for Differential Expressed Proteins Induced by PFOA

Heatmapping, Volcano plot analysis and Venn diagram packaging were used to explore the differentially expressed proteins in PFOA treated group compared with normal control (Fig. 3A, 3B, 3C). Among the 3,327 non-redundant proteins, 112 proteins were significantly upregulated and 80 proteins were downregulated. Significantly changed proteins are shown in the Volcano plot, where the cut $\log_2$ (Fold

Change) was set at 1 and the cutoff P value was 0.05 (Fig. 3B). Among the differentially expressed proteins, upregulated proteins are listed in Table 2 and supplementary table S4; while the downregulated proteins are listed in Table 3.

To further characterize these differentially expressed proteins, we performed GO function annotation analysis via The Gene Ontology (GO) knowledgebase (http://geneontology.org/). Results show that the upregulated and downregulated proteins are mainly involved in the following three biological processes; cellular processes, metabolic process and single – organism processes, and that these processes are localized primarily within the cellular component. When classifying differential proteins by molecular function we find that they are primarily associated with binding and catalytic activity (Fig. 4A, B).

KEGG Pathway analysis (http://www.kegg.jp/kegg/pathway.html) was also used to determine the involvement of differentially expressed proteins in metabolic and cell signaling pathways. The upregulated proteins were primarily involved in peroxisome, PPAR signaling pathway, fatty acid degradation and fatty acid metabolism; while the downregulated proteins were involved in chemical carcinogenesis, biosynthesis of amino acids and drug metabolism (Fig. 5).

Pathway analysis of differentially expressed proteins identified in the rat livers

Utilizing Ingenuity Pathway Analysis software, eight proteins (ACOX1, ACOX2, ACOX3, ACSL1, EHHADH, GOT2, MTOR and ACAA1) were found to be related to oxidation of fatty acid. (Fig. 6A). Two proteins (ASS1 and CPS1) were found to be associated with urea cycle disorder (Fig. 6B).

Effects of PFOA on urea synthesis related genes

To investigate the toxic effects of PFOA on urea synthesis related genes, we surveyed the transcription levels of three genes (*ASL*, *ASS1* and *CPS1*) of key enzymes related to urea cycle using qRT PCR. Compared with the control group, the transcriptional levels of *ASL* remained unchanged (Fig. 7A), however, the mRNA transcriptional levels of *ASS1* and *CPS1* were significantly downregulated in the PFOA exposed groups in a dose-dependent manner (Fig. 7B,C). These results are consistent with the proteomic results.

ASS1 and CPS1 are significantly reduced by PFOA exposure

Expression levels of ASS1 and CPS1 were further verified via western blot. Results show expression levels of ASS1 and CPS1 are significantly downregulated in the PFOA exposed groups in a dose-dependent manner (Fig. 8A, B). These results are also consistent with the proteomic results.

Discussion

In the present study, the physiological effects of PFOA exposure and its role in liver toxicity was investigated in rat models. We hereby demonstrate that PFOA exposure produces significant body weight loss and liver swelling after 14 days exposure. These results are consistent with previous studies of PFOA exposure experiments in rodents (Lau et al. 2007; Starkov and Wallace 2002). Furthermore, we find that PFOA exposure has a significant effect on urea metabolism. Rats exposed to PFOA have reduced urea

concentration in urine compared with normal control rats. On the other hand, PFOA exposed rats presented with high urea concentration in the sera. A high urea content in serum rather than in urine may suggest that PFOA exposure either decreases the ability of the liver to metabolize urea, or that urea may leak into blood stream due to the hepatocyte damage.

Investigation of sera biochemistry reveals that levels of ALT, ALP and UREA increased significantly after PFOA exposure. The increased level of ALT and ALP implies that PFOA exposure contributes to liver damage and metabolic disfunction in rats. Levels of TG and TC were significantly decreased in the serum of treatment group suggesting a reduction in metabolic processes. Additionally, the levels of HDL-C and LDL-C were also decreased. A search of KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathway and MetaboAnalyst metabolic pathway found that these indicators are involved in bile acid metabolic pathways and steroid and steroid hormone synthesis pathways.

iTRAQ-based quantitative proteomics were utilized to define the proteomic changes in rat livers after 14 days of PFOA exposure. Totally, 2,868 proteins were identified by MS, among which, 112 proteins were significantly upregulated and 80 proteins were downregulated. Two enzymes identified through quantitative proteomics analysis, ASS1 and CPS1, were found to be closely related to urea metabolism. The differential expression of ASS1 and CPS1, was then confirmed in western blotting experiments. Confirming the downregulation of the enzymes involved in urea synthesis as a result of PFOA exposure provides potential targets for future intervention and treatment of PFOA toxicity.

Additionally, quantitative proteomics experiments also identified 8 differentially expressed proteins (ACOX1, ACOX2, ACOX3, ACSL1, EHHADH, GOT2, MTOR and ACAA1) all related to oxidation of fatty acid. ACOX1, ACOX2 and ACOX3 are enzymes related to the fatty acid beta-oxidation pathway, which catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs. ACSL1 is an enzyme responsible for the conversion of free long-chain fatty acids into fatty acyl-CoA esters, and thereby plays a key role in lipid biosynthesis and fatty acid degradation. EHHADH is a bifunctional enzyme that is one of the four enzymes of the peroxisomal beta-oxidation pathway. ACCAA1 is a protein involved in the beta-oxidation system of the peroxisomes. The upregulation of these proteins in the livers of PFOA exposed rats implies an increase in liver fatty acid oxidation. This result is consistent with our finding of low levels of TG present in rat liver and sera. Previous studies have also suggested that PFOA exposure may cause accelerated fatty acid oxidation (Chen et al. 2020; Kudo et al. 2006; Yu et al. 2016). In our study, urea-cycle enzymes ASS1 and CPS1 were also downregulated after PFOA exposure, implying that urea synthesis is decreased in liver. Therefore, PFOA exposure accelerated the β-oxidation of fatty acid in the liver of rats, and at the same time inhibited the synthesis of urea in the liver.

Conclusion

In summary, after 14 days of PFOA exposure, rat livers displayed significant liver swelling and aberrant levels of TG, TC, HDL-C, LDL-C and urea. iTRAQ-based quantitative proteomics revealed that deregulated proteins ACOX1, ACOX2, ACOX3, ACSL1, EHHADH, GOT2, MTOR and ACAA1 are all related to oxidation of

fatty acid while ASS1 and CPS1 are associated with urea cycle disorder. Overall, this study provides insight into specific mechanisms of hepatotoxicity as a result of PFOA exposure.

Declarations

Authors' contributions H.L., and L.W., are co-senior authors. H.L., and L.W. designed the study; H.L., FF.L., YB.Z., HX.C., and L.W. carried out the experimental work and analyzed the results; H.L., N.G., S.F. and L.W. wrote the manuscript. All co-authors have edited the manuscript and approved the final version.

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Data availability All data generated or analyzed during this study were included in this published article, Supplementary table S1, Supplementary table S2, Supplementary table S3, Supplementary table S4 were availble from Springer link.

Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

Ethical approval All authors declared that they had no known competing financial interests or personal relationships that seemed to affect the work reported in this article. All authors followed the ethical responsibilities of this journal.

Consent to participate and publish All authors participated and approved the final manuscript to be published.

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Tables

Table 1. Alteration of serum biochemical levels (n = 7) after PFOA exposure, data are mean±SE, significantly different from control group.

	Ctrl	1.25 mg/kg/d	5 mg/kg/d	20 mg/kg/d	F value	*P* value
ALT	27.81±2.51	35.60±2.41	31.47±1.96	56.00±12.00**	3.96	0.02
ALT/AST	0.28±0.04	0.29±0.02	0.26±0.02	0.46±0.02**	13.13	0.00
ALP	159.14±11.33	192.29±17.21	203.29±13.67*	210.71±12.82*	2.67	0.07
Urea	7.01±0.49	6.41±0.45	6.26±0.32	8.83±0.44**	7.5	0.00
TG	0.72±0.11	0.55±0.06	0.46±0.04*	0.55±0.09	1.88	0.16
TC	1.42±0.10	1.07±0.12	0.97±0.11*	1.59±0.22	3.93	0.02
HDL - C	0.96±0.07	0.83±0.08	0.73±0.09	1.06±0.11	2.69	0.07
LDL - C	0.25±0.03	0.13±0.02	0.16±0.03	0.38±0.11	3.63	0.03

*p < 0.05, **p < 0.01

Table 2. Lists of upregulated Proteins (higher than 2 fold) Identified by iTRAQ in rat livers after 20 mg/kg/day PFOA Exposure for 14 Days

Accession	Description	Unique Peptides	Fold changes	*p* value
P07896	Peroxisomal bifunctional enzyme	51	3.76	0.000
O88267	Acyl-coenzyme A thioesterase 1	3	3.73	0.000
P07871	3-ketoacyl-CoA thiolase B, peroxisomal	2	3.53	0.000
O55171	Acyl-coenzyme A thioesterase 2, mitochondrial	5	3.42	0.000
D4A317	Protein Ccdc18	1	3.19	0.000
P23965	Enoyl-CoA delta isomerase 1, mitochondrial	14	3.15	0.000
A0A0H2UI21	Carnitine O-acetyltransferase	24	3.10	0.000
P07872	Peroxisomal acyl-coenzyme A oxidase 1	30	3.08	0.000
A0A140UHW7	Cytochrome P450 2B1	5	3.07	0.000
P13601	Aldehyde dehydrogenase, cytosolic 1	13	2.99	0.000
P08516	Cytochrome P450 4A10	14	2.96	0.000
Q99N59	Alpha-amylase	2	2.82	0.000
P16970	ATP-binding cassette sub-family D member 3	23	2.81	0.000
Q66H58	von Willebrand factor A domain-containing protein 9	1	2.76	0.000
P20817	Cytochrome P450 4A14	5	2.76	0.000
A0A0G2JSR8	Cytochrome P450, family 17, subfamily a, polypeptide 1	3	2.64	0.009
P51647	Retinal dehydrogenase 1	13	2.63	0.000
P20816	Cytochrome P450 4A2	6	2.61	0.003
D3ZSE3	Protein Acot6	1	2.60	0.034
A0A0G2K1S6	Malic enzyme	18	2.56	0.000
Q95567	Mature alpha chain of major histocompatibility complex class I antigen (Fragment)	1	2.49	0.001
A0A0G2K455	Carboxylic ester hydrolase	3	2.47	0.001
O70597	Peroxisomal membrane protein 11A	7	2.39	0.000
Q62651	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	13	2.38	0.000
G3V734	2,4-dienoyl CoA reductase 1, mitochondrial, isoform CRA_a	13	2.36	0.000
P80299	Bifunctional epoxide hydrolase 2	18	2.33	0.037
A0A140UHX9	Protein Ccz1b	2	2.27	0.005
A0A0G2JYI5	Monoglyceride lipase	10	2.25	0.000
P79599	Mature alpha chain of major histocompatibility complex class I antigen (Fragment)	1	2.20	0.002
F1LWL5	Protein Gemin4	1	2.20	0.006
Q5DT04	UDP-glucuronosyltransferase	7	2.19	0.002
F8WG67	Acyl-CoA thioesterase 7, isoform CRA_a	7	2.18	0.001
A0A0G2JT98	Cytochrome P450 3A2	2	2.16	0.004
P17764	Acetyl-CoA acetyltransferase, mitochondrial	23	2.15	0.002
Q6T5F0	UDP-glucuronosyltransferase	1	2.14	0.000
P22791	Hydroxymethylglutaryl-CoA synthase, mitochondrial	29	2.13	0.000
P05183	Cytochrome P450 3A2	8	2.11	0.002
Q4V8F9	Hydroxysteroid dehydrogenase-like protein 2	12	2.10	0.000
D3ZIQ1	Protein Acot4	7	2.10	0.000
O70253	Muscle carnitine palmitoyltransferase I	5	2.08	0.015
B2RZC0	Aig1 protein	1	2.07	0.001
P24464	Cytochrome P450 4A12	1	2.07	0.000
P97524	Very long-chain acyl-CoA synthetase	3	2.05	0.000
B2RYW4	Mitochondrial ribosomal protein L53	2	2.05	0.005
Q8VHK0	Acyl-coenzyme A thioesterase 8	9	2.02	0.000

Table 3. Lists of downregulated Proteins Identified by iTRAQ in rat livers after 20 mg/kg/day PFOA Exposure for 14 Days

Accession	Description	Unique Peptides	Fold changes	*p* value
Q6I7R1	Dehydrogenase/reductase (SDR family) member 7	2	0.11	0.001
F7ET54	Protein LOC298116	2	0.24	0.002
M3ZCQ0	Sulfotransferase	12	0.27	0.001
F1LRV6	GMP reductase	1	0.32	0.008
Q4V8I9	Protein Ugp2	20	0.37	0.004
Q07523	Hydroxyacid oxidase 2	16	0.39	0.003
B6ID08	Metallothionein	3	0.41	0.031
F1M0G0	Protein Rgs17	1	0.43	0.001
Q03336	Regucalcin	20	0.43	0
P14141	Carbonic anhydrase 3	13	0.44	0.001
A0A0G2K950	Protein Papss2	23	0.46	0.001
P10867	L-gulonolactone oxidase	1	0.48	0.024
P04182	Ornithine aminotransferase, mitochondrial	16	0.48	0.029
A0A0G2JWH3	Condensin complex subunit 1	1	0.49	0.026
P04799	Cytochrome P450 1A2	20	0.49	0.001
Q4QQW7	Cytochrome P450, family 2, subfamily c, polypeptide 7	10	0.49	0.029
P16232	Corticosteroid 11-beta-dehydrogenase isozyme 1	12	0.5	0
Q5BK21	Protein Tm7sf2	3	0.51	0
P07379	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	23	0.51	0.013
P09034	**Argininosuccinate synthase**	**26**	**0.55**	**0**
P04905	Glutathione S-transferase Mu 1	12	0.55	0.003
Q569D0	Sulfotransferase	2	0.55	0.006
A0A0G2JSI0	Dimethylaniline monooxygenase [N-oxide-forming]	20	0.57	0.011
A0A0H2UHH2	Serum amyloid P-component	6	0.57	0
P09606	Glutamine synthetase	21	0.57	0
P36365	Dimethylaniline monooxygenase [N-oxide-forming] 1	19	0.57	0.004
P28037	Cytosolic 10-formyltetrahydrofolate dehydrogenase	49	0.57	0.001
B1WBN9	Pyruvate kinase	24	0.58	0
Q6AZ33	Biliverdin reductase A	1	0.58	0.006
D3ZTP0	10-formyltetrahydrofolate dehydrogenase	32	0.58	0.006
P17988	Sulfotransferase 1A1	16	0.58	0.003
P24008	3-oxo-5-alpha-steroid 4-dehydrogenase 1	3	0.58	0.001
A0A0G2K2P4	Protein Cyp2t1	3	0.59	0.007
P70552	GTP cyclohydrolase 1 feedback regulatory protein	2	0.59	0.011
D4AE49	Protein Skiv2l2	1	0.59	0.012
P49890	Estrogen sulfotransferase Ste2	5	0.6	0.004
P49889	Estrogen sulfotransferase, isoform 3	3	0.6	0.035
F1LQS6	RCG61833	27	0.61	0
Q6AZ23	Caspase 6	2	0.61	0.018
P02761	Major urinary protein OS=Rattus norvegicus PE=1 SV=1	3	0.61	0.021
D4A253	Protein LOC100361547	1	0.61	0.022
B6DYQ2	Glutathione S-transferase	13	0.61	0.001
D4A7S6	LOC363020 (Predicted)	1	0.61	0.022
P21213	Histidine ammonia-lyase	24	0.61	0.002
D4A2Y9	Peroxisomal biogenesis factor 13 (Predicted)	2	0.61	0.002
Q64654	Lanosterol 14-alpha demethylase	13	0.62	0
P28492	Glutaminase liver isoform, mitochondrial	8	0.62	0.001
P62329	Thymosin beta-4	4	0.62	0.001
O35394	Prenylated Rab acceptor protein 1	2	0.62	0.036
A0A0G2JSM2	60 kDa lysophospholipase	4	0.63	0
D3ZM33	Protein LOC100362298	9	0.63	0.002
F1LN88	Aldehyde dehydrogenase, mitochondrial	1	0.63	0
D3ZBM3	Ferrochelatase	7	0.63	0.011

Q6IRS6	Fetub protein	9	0.63	0.001
A0A0H2UHB1	3-keto-steroid reductase	3	0.64	0.007
A0A140TAC7	L-gulonolactone oxidase	1	0.64	0.003
Q923M1	Mitochondrial peptide methionine sulfoxide reductase	8	0.64	0.003
A0A0A0MY00	Short/branched chain-specific acyl-CoA dehydrogenase, mitochondrial	12	0.64	0.014
F1MAR6	Protein Prodh1	10	0.64	0.006
P12336	Solute carrier family 2, facilitated glucose transporter member 2	5	0.64	0
P24470	Cytochrome P450 2C23	19	0.64	0.001
D3ZCM7	Protein Tle1	1	0.64	0.004
P52759	Ribonuclease UK114	9	0.64	0.001
P07756	**Carbamoyl-phosphate synthase [ammonia], mitochondrial**	**97**	**0.64**	**0**
P36201	Cysteine-rich protein 2	5	0.64	0
P24090	Alpha-2-HS-glycoprotein	11	0.65	0.007
O88867	Kynurenine 3-monooxygenase	11	0.65	0.003
A0A0G2JZ73	Alpha-1-antiproteinase	15	0.65	0.014
P50554	4-aminobutyrate aminotransferase, mitochondrial	16	0.65	0.001
F1M6G0	Protein RGD1565693	1	0.65	0.012
Q6P691	Protein C2cd2	1	0.65	0.001
Q9QXT3	N-acetyltransferase 8	2	0.65	0.008
Q6AYW2	Phenylalanine hydroxylase	20	0.66	0.004
D3Z9U2	CD163 antigen (Predicted)	4	0.66	0
Q6AYC4	Macrophage-capping protein	3	0.66	0.005
G3V8G5	Golgi apparatus protein 1	2	0.66	0.025
Q4KM35	Proteasome subunit beta type-10	4	0.66	0.001
B2RYT7	Haloacid dehalogenase-like hydrolase domain containing 3	9	0.66	0
P09811	Glycogen phosphorylase, liver form	49	0.66	0.007
A0A0G2K7T2	Bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase	19	0.66	0.026

Figures

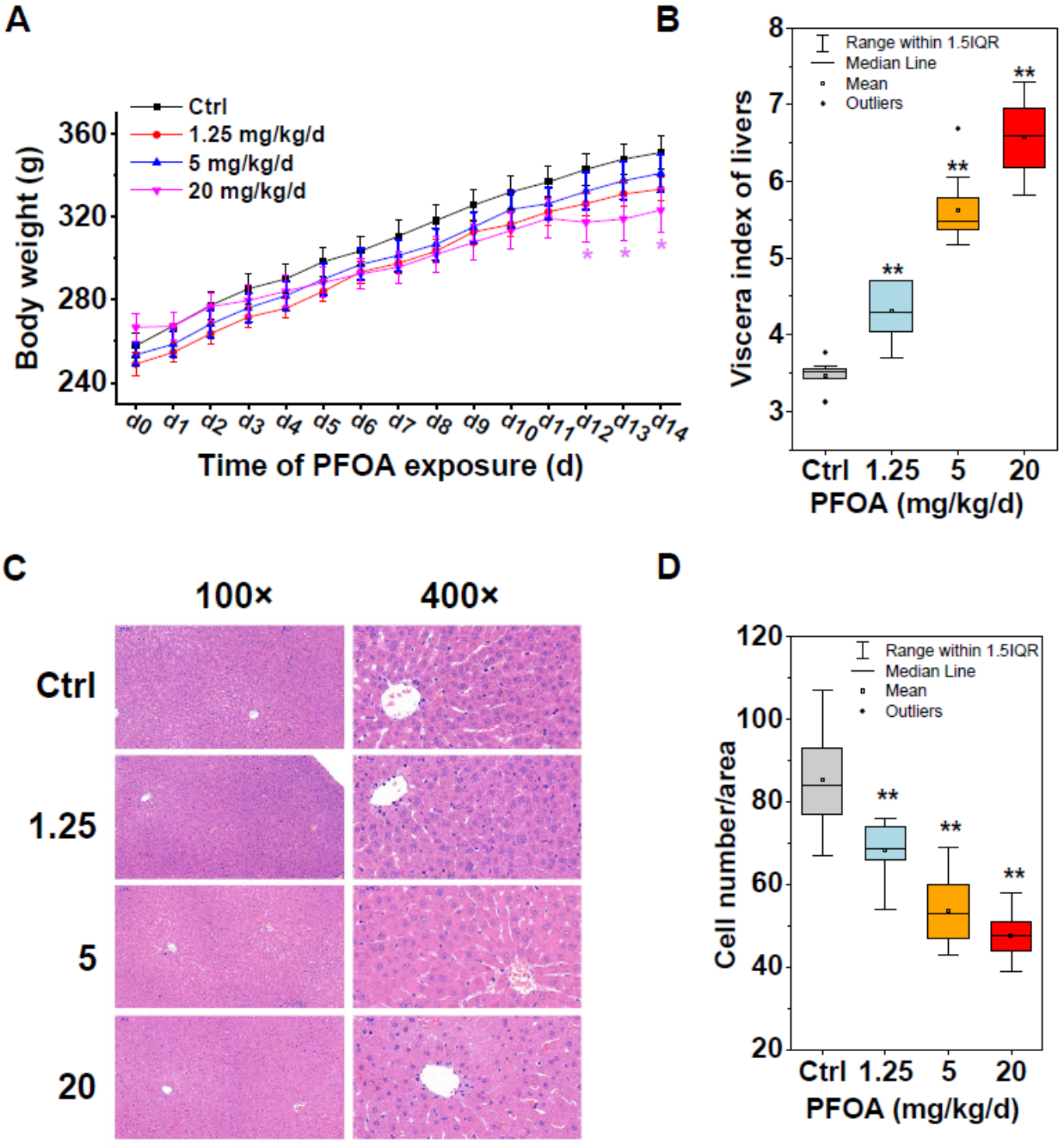

Figure 1

PFOA exposure can cause body weight to lose and significant liver Swelling. A. Body weight gain during PFOA exposure for 14 days in each group. B. Organ index of liver (relative liver weight) was significantly increased by PFOA exposure. C. HE-stained liver slices from PFOA exposed mice compared with normal control (100x and 400x original mag). D. Nuclei number per unit area. Data points represent individual

replicates (C: control; L: 1.25 mg/kg/d; M: 5 mg/kg/d; H: 20 mg/kg/d). Mean ± SEM; n = 10; *p < 0.05; **p < 0.01 (control group vs. PFOA treated groups).

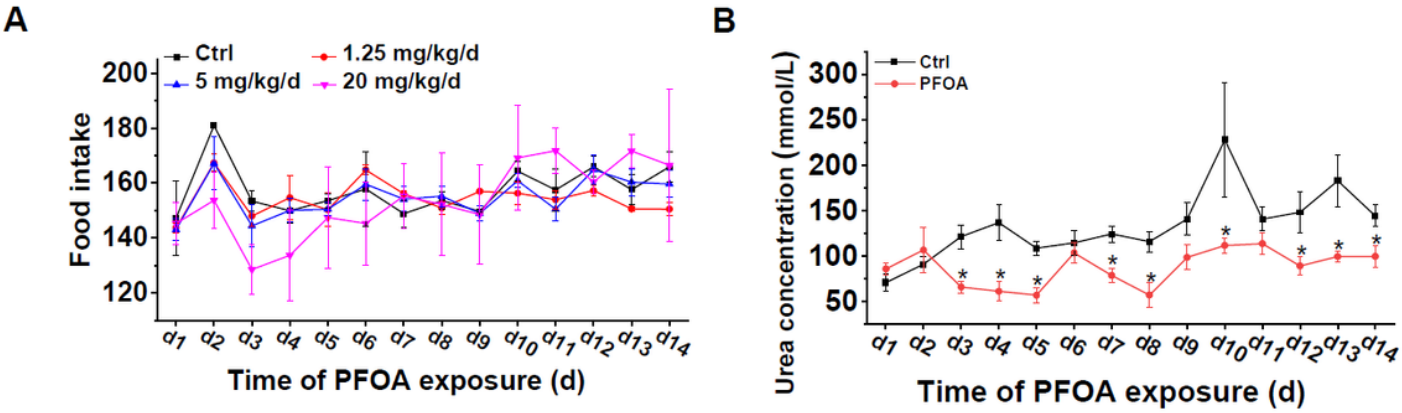

Figure 2

PFOA exposure can affect the urea metabolism. A. PFOA exposure does not change daily food intake in rats. B. Urea concentration in rats' urine decreased significantly after PFOA exposure. Mean ± SEM; n = 5; *p < 0.05; **p < 0.01 (control group vs. PFOA treated groups).

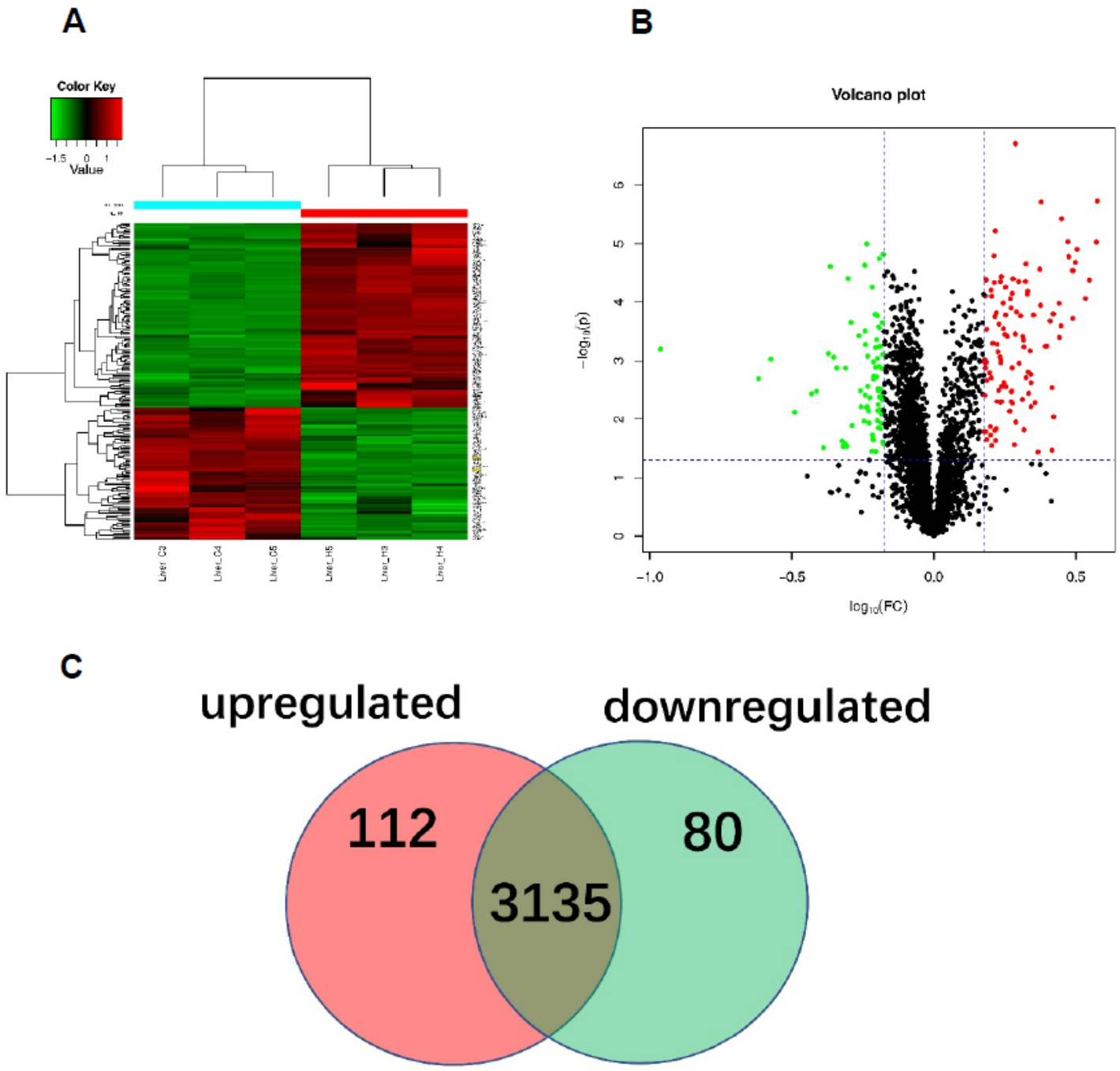

Figure 3

Proteomics analysis of rat liver tissues after PFOA exposure using isobaric tags for relative and absolute quantification (iTRAQ). A. Heatmap of identified proteins. Red colour refers to upregulated proteins, green colour refers to down regulated proteins. B. Volcano plot of identified proteins. C. Venn diagram shows the overlap of upregulated, downregulated and total identified proteins, of which, 112 proteins were significantly upregulated and 80 proteins were downregulated.

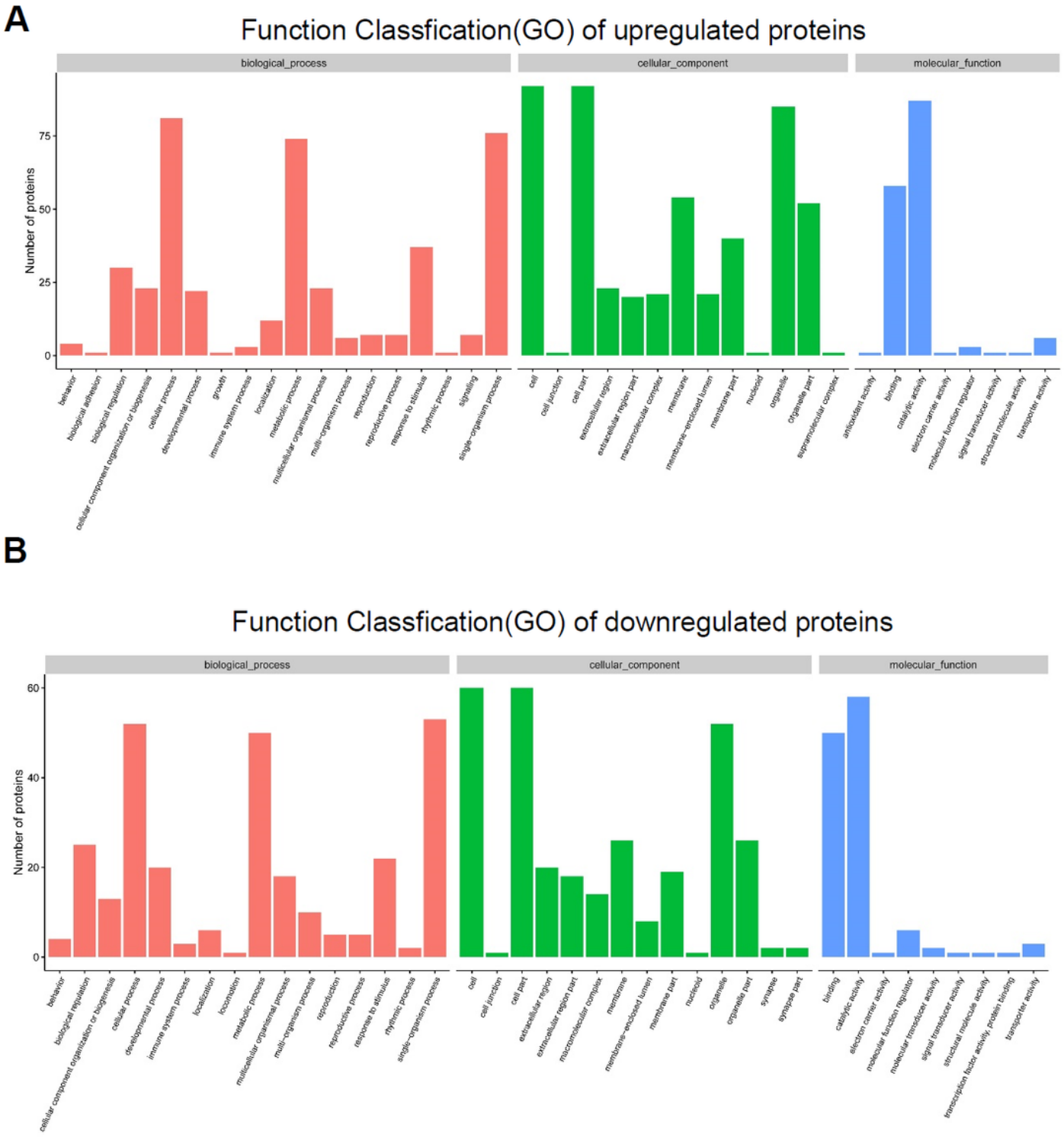

Figure 4

Gene Ontology (GO) analysis of different expressed proteins in rat liver tissues after PFOA exposure. A. Function classfication of upregulated proteins. B. Function classfication of downregulated proteins.

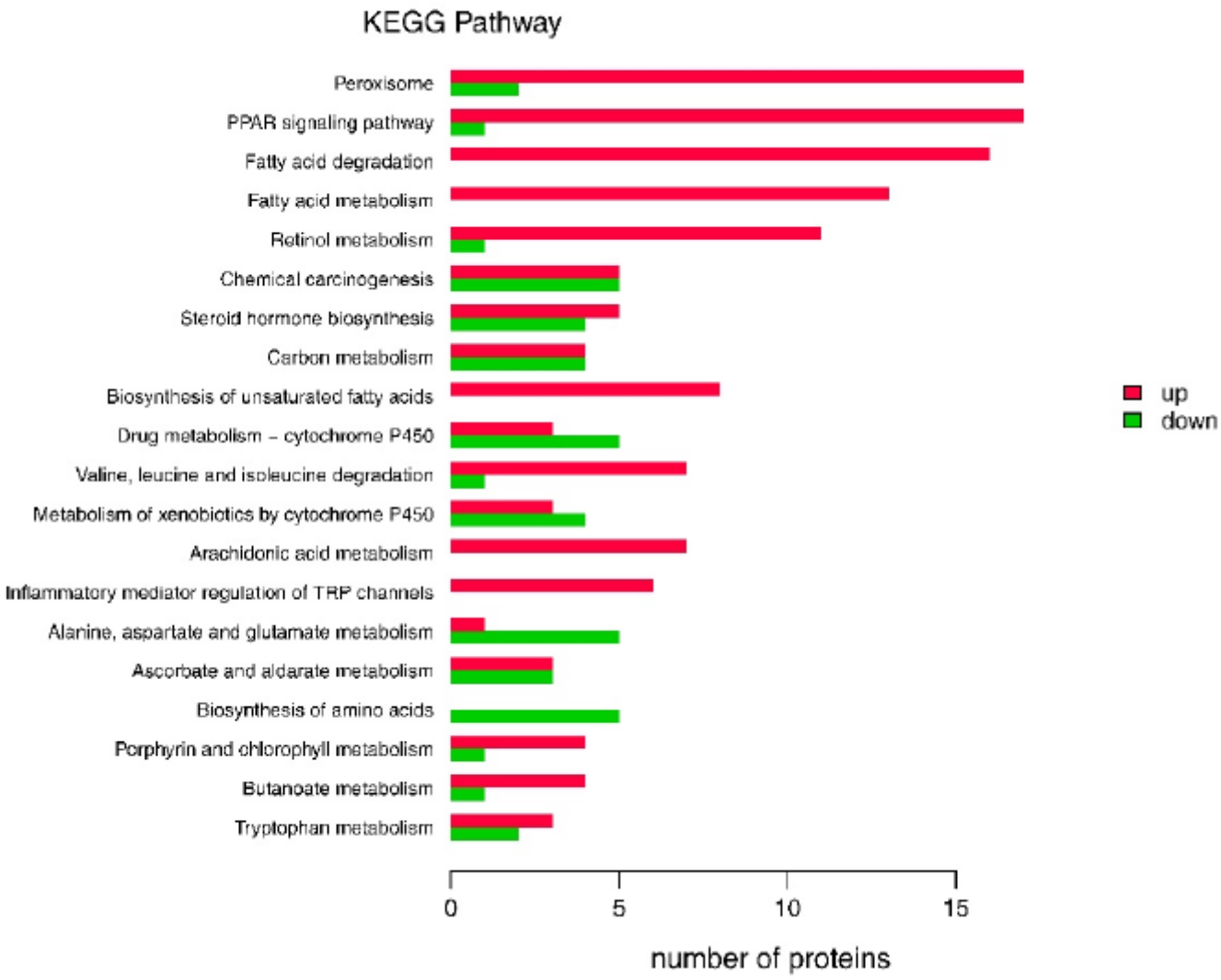

Figure 5

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of different expressed proteins in rat liver tissues after PFOA exposure.

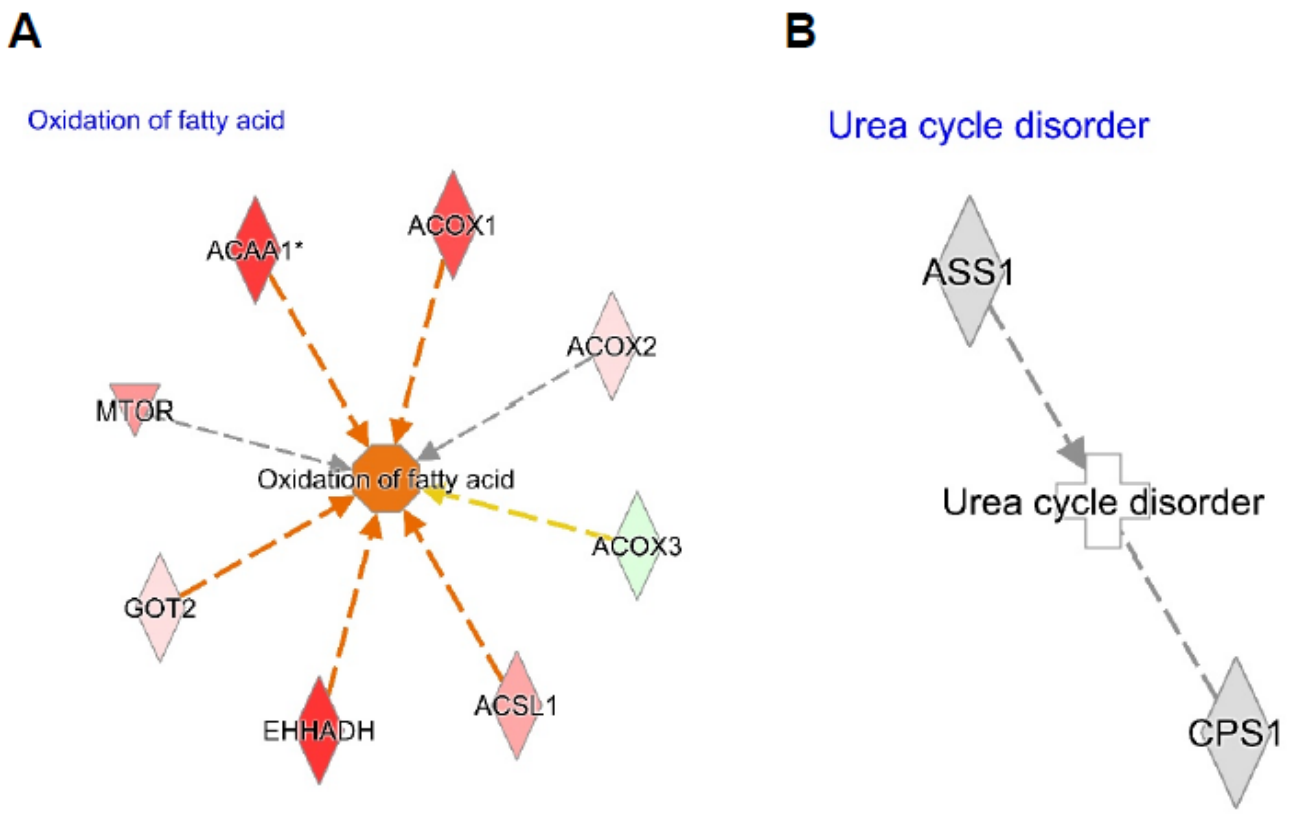

Figure 6

Ingenuity Pathway Analysis (IPA) of some interesting DEPs (different expressed proteins). A. Network of different expressed proteins related to oxidation of fatty acid. B. Network of different expressed proteins related to urea cycle disorder.

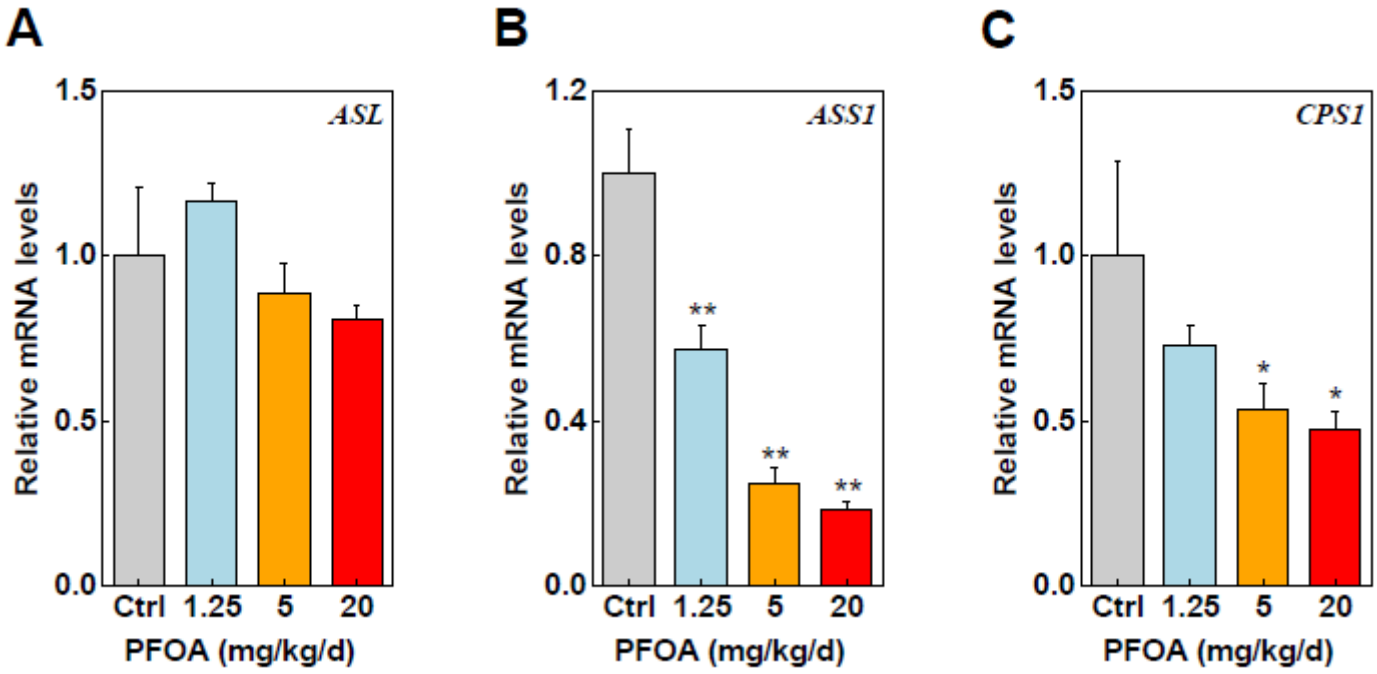

Figure 7

Quantitative RT-PCR analysis of rat liver mRNA transcription levels of control and PFOA treated groups at various concentrations. Mean ± SEM; n = 6 *p < 0.05; **p < 0.01 (control group vs. PFOA treated groups). Asl: Argininosuccinate Lyase; Ass1: Argininosuccinate Synthase 1; Cps1: Carbamoyl-Phosphate Synthase 1.

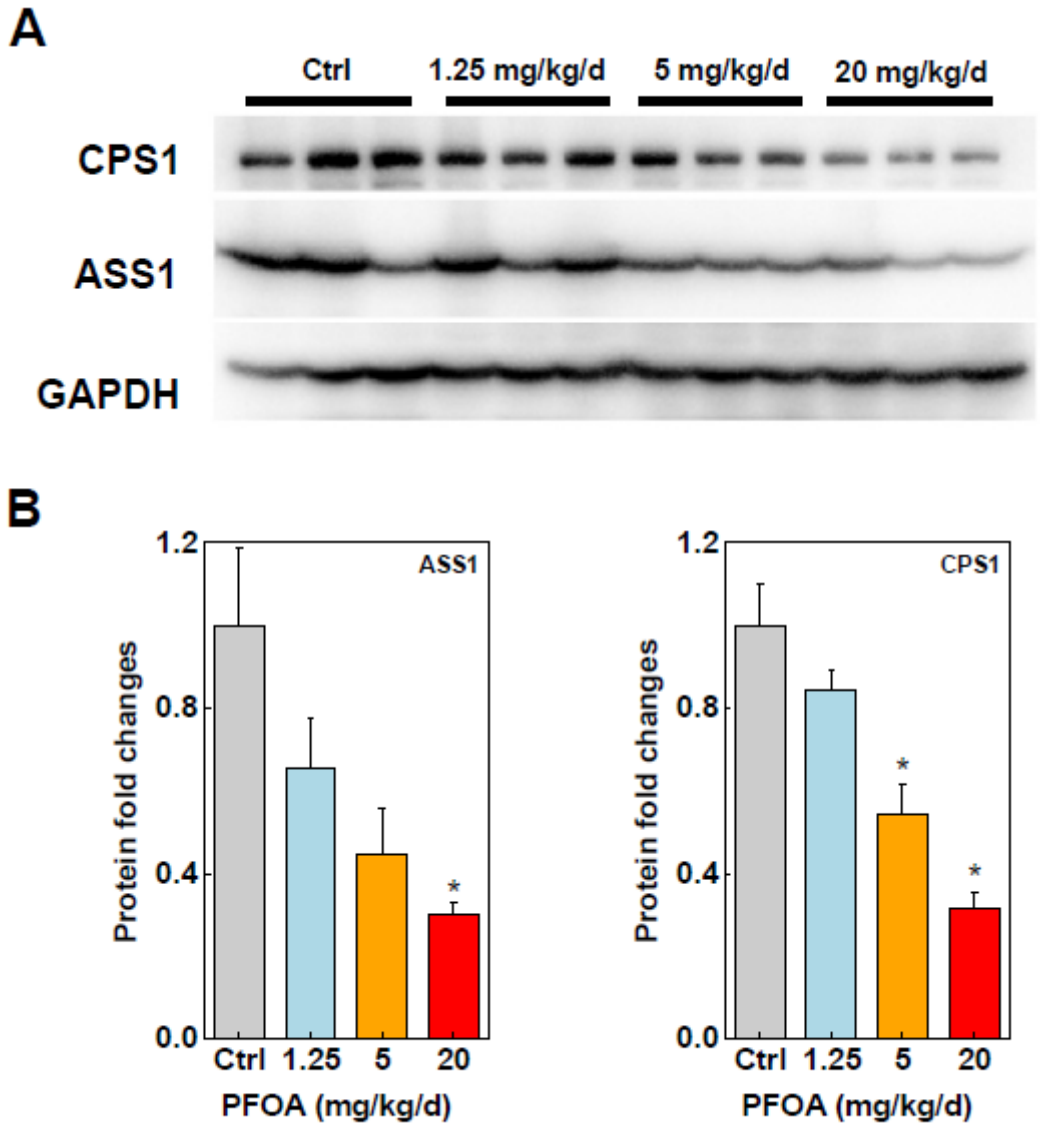

Figure 8

Western blot analysis of rat liver protein expression levels of control and PFOA treated groups at various concentrations. A. Protein levels of CPS1 and ASS1 in rat livers after PFOA treatment. Protein intensities were normalized to the corresponding internal reference protein GAPDH level. B. Results from densitometry analysis of the western blots in A. Mean ± SEM; n = 6 *p < 0.05; **p < 0.01 (control group vs. PFOA treated groups).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.pdf
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- TableS3.pdf
- TableS4.pdf